Journal of Apicultural Research and Bee World 48(4): 256-262 (2009)
DOI: 10.3896/IBRA.1.48.4.05
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ORIGINAL RESEARCH ARTICLE

Feeding essential oils and 2-heptanone in sugar syrup and liquid protein diets to honey bees (*Apis mellifera* L.) as potential Varroa mite (*Varroa destructor*) controls

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Received 27 March 2009, accepted subject to revision 28 May 2009, accepted for publication 30 July 2009.

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Summary

Essential oils were fed to honey bees to determine whether the components were absorbed into bee larvae. The oils were added in either sucrose syrup (origanum and 2-heptanone) or in a liquid protein diet (origanum oil, cinnamon oil, thymol, and 2-heptanone), because sugar and protein sources are differentially utilized in food fed to larvae of different ages. The volatiles emitted by isolated larvae from oil-supplemented colonies were sampled at three different ages (Day 4, Day 6 and Day 9) by Solid Phase Micro-Extraction (SPME) and analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The only oil volatiles recovered in larvae were the origanum oil components carvacrol and thymol; neither 2-heptanone nor the cinnamon oil components were detected in any larvae. For larvae from colonies fed oil-supplemented sugar syrup, carvacrol volatiles were emitted at higher amounts in younger larvae (Day 4) than in older larvae (Day 9). In contrast, carvacrol and thymol volatiles were detected only in older larvae (Day 6 and Day 9) in colonies reared on oil-supplemented liquid protein diet. Carvacrol was also detected in the cocoons of Day 9 larvae from colonies fed oil-supplemented liquid protein diet, but not oil-supplemented sugar syrup diet. We believe that the age-related differences in oil incorporation by bee larvae reflect the relative importance of supplemental sugars and protein as food sources for bee larvae of these ages. Supplementation in a liquid protein diet represents a more efficacious route for the incorporation of origanum oils in fifth instar bee larvae targeted for invasion by *Varroa destructor* mites.

Alimentación con aceites esenciales y 2-heptanona en sirope de azúcar y dietas de proteína líquida a abejas (*Apis mellifera* L.) como control potencial contra el ácaro Varroa (*Varroa destructor*)

Resumen

Se añadieron aceites esenciales para alimentar a abejas para determinar si los componentes son absorbidos por las larvas. Los aceites se añadieron al sirope de azúcar (orégano y 2-heptononа) o a una dieta líquida con proteínas (aceite de orégano, aceite de canela, timol y 2-heptanona) porque las fuentes de azúcar y proteínas se usan de forma diferente en la alimentación de las larvas de diferente edad. Los volátiles emitidos por larvas aisladas de colonias suplementadas con aceite se muestrearon en tres momentos diferentes (día 4, día 6 y día 9) mediante cromatografía de gases y espectometría de masas (CG-EM). Los únicos aceites volátiles recuperados en las larvas fueron los componentes carvacrol y timol del aceite de orégano, no se detectaron componentes de la 2-heptanona ni del aceite de canela en ninguna larva. En larvas de colonias alimentadas con sirope de azúcar suplementado con aceite, se emitieron cantidades mayores de volátiles de carvacrol en larvas jóvenes (día 4) que en las mayores (día 9). Por otro lado, volátiles de carvacrol y timol se detectaron sólo en larvas mayores (días 6 y 9) en colonias criadas con dietas de proteína líquida suplementada con aceite, pero no con la dieta de sirope de azúcar suplementada con aceite. Creemos que las diferencias relacionadas con la edad en la incorporación de los aceites a las larvas reflejan la importancia relativa de suplementar azúcar y proteína como fuente de alimento para larvas de esas edades. El suplemento en una dieta de proteína líquida representa una ruta más eficaz para la incorporación de aceites de orégano en larvas de quinto instar objeto de la invasión de ácaros *Varroa destructor*.

Keywords: essential oils, honey bee larvae, liquid protein diet, SPME, *Varroa destructor*

Introduction

Honey bees colonies are under attack by the ectoparasitic mite *Varroa destructor* (Anderson and Trueman, 2000) with losses of managed bee colonies ranging from 25 to 80% (Finley *et al.*, 1996). More recently, researchers studying "Colony Collapse Disorder"(CCD) have blamed colony deaths, at least in part, on *V. destructor* and the viruses that it carries (van Engelsdorp *et al.*, 2007; Caron, 2009). Therefore, efforts to control this mite are critical, especially now that in many areas *V. destructor* is resistant to the two acaricides used to control it; pyrethroids (e.g. fluvalinate) and organophosphates (e.g. coumaphos) (Lodesani *et al.*, 1995; Elzen *et al.*, 1999; Elzen and Westervelt, 2002). Furthermore, these acaricides accumulate in beeswax and other hive products because of their lipophilic properties (Wallner, 1999; Martel *et al.*, 2007; Caron, 2009).

It is now crucial that beekeepers have access to new control tactics that do not harm bees or contaminate hive products. Ethereal plant oils, also known as essential oils, and other volatile compounds have been used to control bee mites with some success (Ariana *et al.*, 2002; Calderone *et al.*, 1995; Colin *et al.*, 1997; Imdorf *et al.*, 1995; Ruffinengo *et al.*, 2007; Sammataro *et al.*, 2004). Recently, two commercial essential oil products (Apiguard® and ApiLife Var®) for *V. destructor* control have become available in the USA. Both products kill phoretic mites on adult bees through the slow release (Imdorf *et al.*, 1999) via sublimation during routine hygienic and trophallaxis bee behaviour, of thymol and other essential oil vapours from crystals embedded in a polyacrylic acid gel matrix. Thymol has been approved by the Environmental Protection Agency as an acaricide against *V. destructor,* and no adverse effects on humans have been found (EPA Code 080402). Effective vapour coverage throughout the colony is achieved by the exchange of thymol-impregnated gel fragments between workers during routine hygienic and trophallaxis behaviours.

The major drawback of these vapour-phase oil products is the high variation in oil volatility in the hive environment with fluctuating ambient temperatures. At low ambient temperatures, the vapour pressure of thymol is too low to allow for sufficient release of a controlling dose of vapours, and at the same time, the worker activities that distribute the essential oils around the hive decrease appreciably at reduced hive temperatures. In addition, vapour-phase applications by gel release provide only incidental protection for the fifth instar bee larvae just before capping, the life stage targeted for cell invasion by phoretic mites. These problems, combined with the fact that vapours do not kill mites already enclosed in with capped brood, make mite control with essential oils by a vapour phase delivery system unpredictable.

In this project, we evaluated medication by feed supplementation of adult bees as an alternative method for the indirect delivery of essential oils to honey bee larvae. Feed supplementation has long been used by beekeepers as a technique to broadly introduce a variety of stable compounds to bee colonies, including antibiotics, fungicides, and nutritional supplements. Bee larvae acquire supplemental medications through frequent feeding by adult nurse bees that have either ingested the compounds directly from the supplemental feed or indirectly by trophallaxis with other adults. We believe that feed supplementation could be a particularly effective method for the delivery of essential oil components to the most susceptible larvae, since fifth instar bee larvae consume food and gain weight at a higher rate than any other life stage (Sammataro and Finley, 2007), and are provisioned by nurse bees just before capping.

Our goal was to determine whether the essential oils and other volatile-phase mite treatments introduced in the colony via supplemental feeding delivery systems were incorporated by bee larvae of different ages. We evaluated sugar syrup and liquid protein diets, the two most common liquid diet supplemental methods, separately, since each diet type varies in its utility to larvae of different ages. Because mite control by essential oils occurs through vapour-phase release rather than direct contact, we measured volatile emissions of the oil components from bee larvae as a more accurate indicator of the effective delivery of essential oils to bee brood. Solid Phase Micro-Extraction (SPME) techniques were used in tandem with Gas Chromatography-Mass Spectrometry (GC-MS) analysis to collect and evaluate volatiles emanating from the immature larvae for evidence of essential oil incorporation.

Materials and methods

Experiment 1: to determine whether compounds suspended in sugar syrup solution can be traced in bee larvae.

Experiment 2: to determine whether compounds suspended in liquid protein diet can be traced in bee larvae.

Colonies housed in nucleus hives, five per treatment, and which had ample colony strength and adult populations, were fed exclusively on diets containing treatment components either emulsified in sugar syrup (Experiment 1, November 2004) or suspended in a liquid protein diet (Experiment 2, spring 2005). The protein diet (MegaBee; S.A.F.E., Tucson, AZ, USA) contained no pollen. Essential oils were selected because of their known toxicity to *V. destructor* (Calderone *et al.*, 1995) and their low toxicity and repellency to honey bees. Thymol and its native oil origanum were tested, because thymol is the primary active component of the vapour-phase acaricides Apiguard® (Vita Europe Ltd; Basingstoke, UK) and ApiVarLife® (Chemicals LAIF; Vigonzo, Italy). The ketone 2-heptanone and cinnamon oil were added because of their known properties against *V. destructor* (G. De Grandi-Hoffman, G. Wardell, pers. comm.). Thymol, origanum oil, and cinnamon oil were obtained from Sigma-Aldrich (St. Louis, MO, USA) and two formulations of encapsulated 2-heptanone were supplied by Cerexagri-Nisso LLC (King of Prussia, PA, USA). Supplements were incorporated into both diets by first emulsifying them in water, and then by adding measured amounts to 1:1 sugar: water (w/w) solution to obtain a final concentration of 0.06% for each of the oil compounds in the treatments. Treatment compounds were integrated into the liquid protein diet as part of a 1:1 sugar syrup mixture added to the diet (at 20%) as a feeding stimulant.

In Experiment 1, treatment colonies were fed a sugar syrup diet (1:1 sucrose: water w/w) supplemented with either origanum oil or two formulations of 2-heptanone. For Experiment 2, treatment colonies were fed a liquid protein diet mixture that contained either origanum oil, cinnamon oil, thymol, or the two formulations of 2-heptanone. Control colonies were fed either un-supplemented sucrose syrup (Experiment 1) or liquid protein diet with no added essential oil components (Experiment 2). Bees were provided with supplemental diets for either two weeks (Experiment 1) or one month (Experiment 2) before sampling and allowed to feed *ad libitum* from in -hive feeders throughout the experiment. The length of time was determined as long enough to allow the oils to penetrate the larval cuticle depending on the type of carrier (sugar syrup *vs.* protein diet).

Collection of larvae

To obtain larvae of known ages, queens were caged overnight to obtain combs of uniform age from which three different ages of larvae were later sampled. All queens were of Italian origin (*Apis mellifera ligustica*), purchased from Big Island Queens (Captain Cook, Hawaii, USA). Five larvae were collected from each treatment colony on the 4th (first instar), 6th (third instar), or 9th (fifth instar) day after oviposition, following procedures developed by Finley and Sammataro (2008). Day 4 larvae are just hatched from the egg and are fed copiously with food by the nurse bees. By the 9th day, the larvae are much bigger, have finished feeding, and are about to be capped over to pupate. It is at this stage that female *V. destructor* invade to lay their own eggs.

Each frame of known aged brood was removed from the colony and brought into the lab for sampling. To minimize larval stress during brood extraction, the laboratory was maintained at 28.3-31.1ºC and 35-45% RH to simulate broodnest conditions. Live brood was extracted intact from cells with grafting tools and forceps after pushing away the wax cell walls. All tools used to collect brood were cleaned with 70% ethanol, rinsed in distilled water, and dried with KimWipes (Kimberly-Clark, Roswell, GA, USA) to remove any residues. Only mite-free larvae were used.

Volatile collection

For each volatile sample, five larvae were placed in 7.5 ml (Day 4 larvae) or 20 ml scintillation vials (Day 6 and Day 9 larvae). Each of the sample vials was covered with a strip of aluminium foil, with care being taken to not contaminate the sample with extraneous volatiles from hands, vinyl gloves, or marking pens, and labelled with paper tags and graphite pencils. The sample vials were then stored in an incubator (Caron 6010; Marietta, OH, USA) at 33ºC and 50% RH until the volatiles were ready to be collected (usually within 30 min.). Larval volatiles were collected using a StableFlex™ 65µm polydimethylsiloxane/ divinylbenzene coated SPME fibre (Supelco #57326-U; Bellefonte, PA, USA). The SPME fibre was pushed through the aluminium foil cap and exposed to odours from the larvae for 10 minutes at approximately 28-31°C and 35% RH. Additional experiments with larval volatiles demonstrated that this exposure time was sufficient to establish equilibrium between the SPME fibre and the container atmosphere for each sample. To obtain background volatiles, diet materials (sugar syrup or liquid protein diet) were sampled separately and air blanks (consisting of empty vials) were collected each day before larval volatile samples. Oil standards were collected by adding 1 µl of material to a 20 ml vial and exposing the fibre for 1 second.

GC-MS analytical instrumentation and procedures

Volatile components were identified and quantified by positive ion electron impact gas chromatography mass spectrometry (GC-MS) performed on a Varian 3800 gas chromatograph coupled to a Saturn 220 Ion Trap detector (150 eV; Varian, Inc., Palo Alto, CA, USA). Volatiles absorbed on the SPME fibre were introduced at 250°C in a Varian 1079 injector to a Varian Factor Four VR5 column (30 m x 0.25 mm ID) with helium as the carrier gas at 1 ml/min. Sample components were separated under column oven conditions of 40°C for 3 min, 20°C /min to 100°C, 90°C/min to 200°C, and 20°C/min to 250°C. The split ratio was 1/20 for 0.1 min, then off until 3.0 min and then 1/20 for the duration of the run (19 min).

Compound identity was confirmed by comparing the sample GC retention time with the appropriate standard and the sample mass spectra pattern with National Institute of Standard and Technology (Gaithersburg, MD, USA) and Wiley® 2007 mass spectra libraries and Varian MS Workstation (Walnut Creek, CA, USA). To eliminate interference from background spectra, quantification of volatile components was performed using selective ion filtering of sample chromatograms. For carvacrol and thymol (the major components of origanum oil), the ions selected were 135 and 150 mass: charge ratio (m/z). For cinnamaldehyde (the major component of cinnamon oil), the ions were 77 and 115 m/z, and for 2-heptanone, the ions were 43 and 58 m/z. To estimate the equivalent amounts of carvacrol and thymol present in the samples, a standard curve was made for the volatile release of each oil compound from a buffer solution. Standards for thymol and carvacrol were made as 0, 2, 4, 8, 20 and 40 µM solutions (600 µl) in 10 mM phosphate buffer at pH 7.0 in a 20 ml scintillation vial. The equivalent concentration of thymol and carvacrol present in the samples (in µM) was estimated from the compound peak area as:

[Thymol] µM= Area x 0.0027 + 0.2309; R^2 = 0.9971

[Carvacrol] µM = Area x 0.0026 + 0.0219; R^2 = 0.992

Conversions to amounts (in *ng*) and contents (in *ng* compound/ *mg* insect tissue) of carvacrol and thymol were made by taking into account the volume of the collection container, the molecular weight of thymol or carvacrol (150.22 *g*/mol), and the mass of the larval sample.

Results

Only two of the oil components, carvacrol and thymol, were detected in larvae from the colonies fed supplemental diets. None of the major oil components associated with 2-heptanone formations or cinnamon oil (namely 2-heptanone and cinnamaldehyde) were detected in any of the larvae. Carvacrol and thymol volatiles were emitted by some of the larvae from all colonies fed origanum oil and thymol supplements, but the pattern of incorporation by each age cohort was strongly dependent on the diet medium that carried the supplements.

In Experiment 1, the volatile carvacrol, but not thymol, was detected at higher absolute levels in younger larvae in colonies fed supplemental sugar syrup (Fig. 1). Day 4 larvae had 58 ng carvacrol / larva while Day 9 larvae had 39 ng / larva. The greater carvacrol volatile content of younger larvae is much more apparent when the volatile emissions are corrected for the much smaller size of Day 4 larvae. By contrast, carvacrol and thymol volatiles were

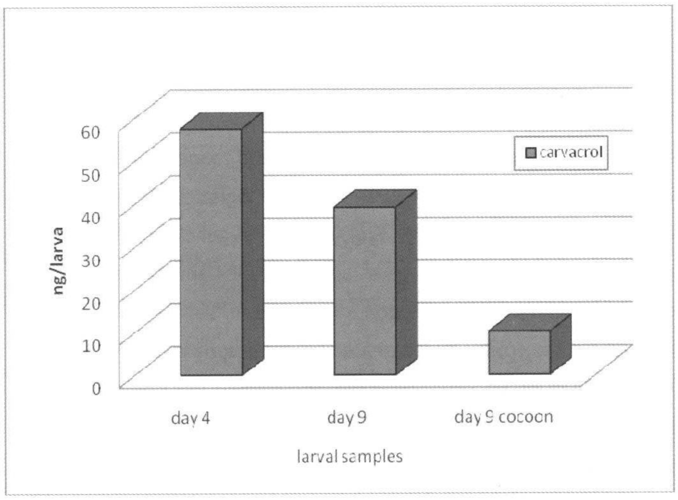

Fig. 1. Experiment 1. Estimated amounts of carvacrol in different ages of bee larvae and cocoons, from colonies fed essential oil supplemented sugar syrup diets. The supplemented sugar syrup diet contained origanum oil and two formulations of 2-heptanone (0.06% for each additive) and the control was only sugar syrup. Only carvacrol was found.

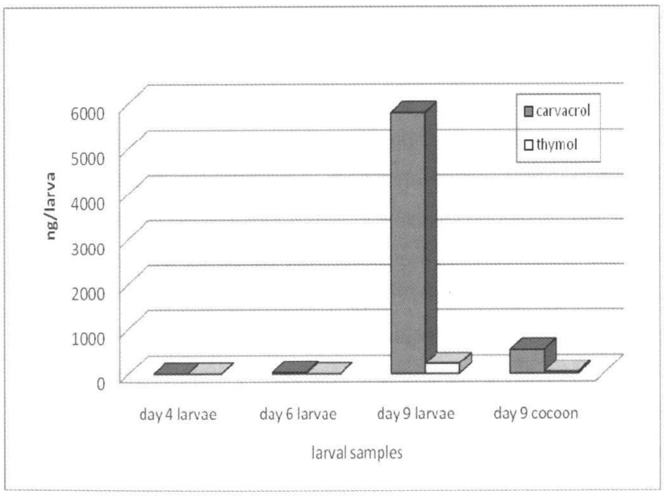

Fig. 2. Experiment 2. Estimated amounts of carvacrol and thymol in different age bee larvae and cocoons from colonies fed essential oil supplemented liquid protein diet. These compounds were the only ones recorded from larvae and cocoons.

detected only in older larvae in colonies fed the supplemental liquid protein diets in Experiment 2 (Fig. 2). While Day 4 larvae had no detectable levels of either compound, Day 6 larvae had 29 ng carvacrol / larva and Day 9 larvae had 5798 ng carvacrol / larva and 227 ng thymol / larva; likewise, both carvacrol and thymol volatile emissions were detected in cocoons obtained from Day 9 larvae (538 ng / larva and 43 ng / larva respectively). The greater carvacrol and thymol content of older larvae is evident even when the contents are adjusted for larval mass. None of the oil component volatiles were recovered from larvae reared in the colonies fed un-supplemented sugar syrup or liquid protein diets.

Discussion

Medication of honey bee colonies by dietary supplements has long been used for the effective delivery of a broad range of products, including vitamin supplements, antibiotics, fungicides, and acaricides (Shimanuki and Knox, 1997). Supplemental sugar syrup and various protein diets have both served as a delivery medium for medications, since they promote the ingestion and sharing of supplements throughout the colony by larval feeding and trophallaxis. For effective medication to occur, however, a diet supplement must persist in an available form long enough to be taken up and spread throughout the colony by the bees. Of the five dietary components added to the supplemental diets, only the origanum oil components carvacrol and thymol were detected in honey bee larvae. Whilst it is not clear why only carvacrol and thymol were successfully incorporated by the bee larvae, limitations of incorporation of the oil components could have arisen at several points. The oil components may not have been completely available to bees consuming the supplementary diets due to separation from the aqueous media. Essential oil components can be difficult to maintain in solution or suspension in aqueous media such as sugar syrup or liquid protein diet due to their lipophilic characteristics and low solubility in water. We noted that the oils were difficult to keep in suspension when not immediately consumed, especially in cold weather when both dietary consumption by the colony and compound solubility in water markedly declined. Even when the oil components are successfully consumed by the bees, the persistence of these volatile compounds can be offset by their continuous loss to the surrounding colony atmosphere.

In particular, 2-heptanone is considerably more volatile than the other oil components (as indicated by its lower boiling point, and hence its utility as an ephemeral alarm pheromone) and may have dissipated more rapidly when exposed. The dietary supplements also could have been metabolized or absorbed differentially by both the adult bees and the bee larvae. Another possibility is that because 2-heptanone is a ketone, it could have reacted to compounds in the bee's system that have amine moiety and thus formed Schiff base type compounds (Kaminskas *et al.*, 2005). The 2-heptanone is probably more reactive towards other biological nucleophilic compounds such as proline, other amino acids and proteins, which are in bee hemolymph in large amounts (Hrassnigg *et al.*, 2003). Our results suggest that carvacrol and thymol may be effective acaricides, in no small part due to their efficient delivery, persistence and duration in the honey bee colony environment.

Our most surprising finding was that younger and older bee larvae acquire carvacrol and thymol in different amounts from sugar syrup and liquid protein supplemental media. These patterns probably reflect changes in the relative importance of various larval food sources over time. Young worker larvae (Day 4) are initially fed brood food, a highly processed secretion consisting of separate clear and milky white components produced in the hypopharyngeal and mandibular glands of worker bees. Worker bees are stimulated to produce brood food by feeding on nectar, honey, or supplemental sugar sources such as sucrose syrup. Brood food contains sugars mixed into the material from a fresh source such as honey, but the proteinaceous fraction of brood food originates primarily in protein previously digested by nurse bees after they first emerged from their brood cells (Winston, 1987). Young worker larvae are therefore more likely to encounter essential oils from fresh, undigested sugar syrup than from previously digested protein supplements. By contrast, older worker larvae (Day 9) are directly fed increasing amounts of protein such as pollen or protein supplements that have not been digested by the nurse bees (Winston, 1987). These fresh protein sources have been minimally processed by the nurse bees and may contain residual oil components that might not survive digestion in an adult bee. Older (fourth and fifth instar) bee larvae are therefore more likely to obtain essential oils from an oil-supplemented pollen or supplemental protein source such as a liquid protein diet, than a sugar source.

Furthermore, oil-supplemented protein diets are heavily consumed by adult worker bees at ages that are most intimately involved with both mites and bee larvae. Newly emerged adult workers gorge themselves on proteinaceous material in part to fully develop the mandibular glands used to feed bee larvae. These workers continue to consume large quantities of protein in their subsequent tasks as nurse bees, in order to directly feed older bee larvae (Winston, 1987). Mites are therefore exposed to supplemental oils in their preferred brood hosts (late fifth instar larvae), their initial phoretic hosts (newly emerged adults), and their preferred phoretic hosts (young nurse bees). Clearly, if the goal is to incorporate origanum oil components into the honey bee life stages that serve as hosts, the oil components should be supplemented into a protein diet rather than sugar syrup diet. In addition, the direct contamination of honey and nectar stores by oil components in supplemental protein diets is limited because only pure sugar sources, not protein supplements, are directly processed into sugar stores (G Wardell, pers. obsv.).

A significant proportion of the targeted fifth instar (Day 9) hosts do not feed just before capping, and therefore appear less amenable to direct medication through diet during the most vulnerable life period. Carvacrol and thymol are, however, persistent enough in fifth instar larvae reared on oil-supplemented liquid protein diets to enter the capping phase. These oil volatiles need not be emitted directly from the larvae themselves, but may originate from other materials enclosed in the cell. Although larvae stop feeding just before capping, their cells are contaminated by faeces and are often provisioned with brood food that may contain supplemental oil components. Our results indicate that fifth instar larvae from oil-supplemented colonies spin cocoons with detectable residues of carvacrol, particularly when the oils had been added to a protein diet. Whilst it is unclear whether the carvacrol residues are acquired directly from contaminated silk, faeces, or indirectly from contact with the larva, brood food, or the cell proper, the residues become enclosed with invading mites and their larval host after capping.

Paradoxically, mites that are not deterred from cell invasion may be killed or disabled by trace oil components once the open cell is capped over. Volatiles released from surfaces in an open cell rarely achieve high local concentrations because the vapour components are constantly lost by diffusion and convection to the much greater volume of the hive environment. By contrast, oil volatiles are largely trapped within the cell volume once the cell is capped over with a relatively impermeable wax cap. Even small traces of oil components can build toward saturation and persist longer, probably due to the slow transit of oil vapours through or into wax. Further studies should investigate whether the small amounts of carvacrol and thymol residues inside capped cells can restrict mite survival and reproductive success without injuring the bee brood host.

Acknowledgements

Dr. J. Hooper of PIMA Research, LLC was extremely helpful in conducting and running the GC/MS, for clarifying the protocols and giving helpful comments for this manuscript. The referees also added many useful comments to this manuscript. This work was partially funded by the Almond Board of California

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